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Investigation of cytogenetic markers in neonatal blood spots and of aberrant protein expression in bone marrow from children with leukemia

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Investigation of cytogenetic markers in neonatal blood spots and of aberrant protein expression in bone marrow from children with leukemia

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ABSTRACT

The general aim of this thesis was to contribute to a better understanding of the development and progression of leukemia in children. Biological markers identified in children with leukemia can contribute to the understanding of leukemic development.

Several studies have traced biological markers associated with leukemia back to a prenatal origin by analyzing neonatal blood spots (NBSs) indicating that these markers are early events in leukemogenesis. The fusion gene *STIL-TAL1* occurs with a frequency of 11-27% at diagnosis of T-cell acute lymphoblastic leukemia (ALL) in children. The developmental timing of this fusion gene is unknown yet, *STIL-TAL1* is exclusively found in T-cell ALL hence could be a key event in development of this type of leukemia.

Response to antileukemic treatment is important for prognosis in leukemia, but also specific molecular cytogenetic markers are important for classification and risk assessment. Mutations in the tumor suppressor gene *TP53* are the most common genetic aberrations in cancer and analysis of altered expression of its protein product p53 has shown to have potential as a predicitve marker for adverse outcome in leukemic patients.

In **scientific paper I**, we investigated if the fusion gene *STIL-TAL1* is present early in life by analyzing NBSs from 38 patients who developed T-cell ALL in childhood. DNA was extracted from the NBSs and analyzed for the fusion gene *STIL-TAL1* by nested polymerase chain reaction and electrophoresis. We could not detect the presence of *STIL-TAL1* in any of the 38 neonatal blood spots from pediatric T-cell ALL patients, indicating that *STIL-TAL1* most probably is a post-natal event in development of T-cell ALL in children

In **scientific paper II, III and IV** we investigated if alterations in expression of specific cell cycle regulating proteins can predict relapse after hematopoietic stem cell transplantation (HSCT). Protein expression of p53 were analyzed in 33 patients with rare myeloid malignancies, 34 patients with acute myeloid leukemia (AML) and in 46 patients with B-cell ALL respectively. Protein expression was analyzed by immunohistochemistry (IHC) on tissue micro arrays. In **study II,** increased expression at diagnosis significantly predicted relapse after HSCT in children with rare myeloid malignancies. In **study III,** we found a higher p53 expression at three to six months after HSCT in the AML patients that relapsed compared to the group of patients who did not relapse. In **study IV** we found that an intense expression of p53 protein within three months after HSCT predicted relapse in patients with

ALL. In summary, we conclude that aberrant protein expression of p53, analyzed by IHC which is a well-established method available at most hematological laboratories, may have potential as a prognostic marker to predict relapse after HSCT in pediatric leukemia.

LIST OF SCIENTIFIC PAPERS

I. Gustafsson B*, **Mattsson K***, Bogdanovic G, Leijonhufvud G, Honkaniemi E, Ramme K, Ford A.M. Origins of *STIL-TAL1* fusion genes in children who later developed paediatric T-cell acute lymphoblastic leukaemia: An investigation of neonatal blood spots. *Shared first authorship. Pediatric Blood & Cancer. 2018 Nov; 65(11):e27310.

II. Honkaniemi E*, **Mattsson K***, Barbany G, Sander B and Gustafsson B. Elevated p53 protein expression; a predictor of relapse in rare chronic myeloid malignancies in children? *Shared first authorship. Pediatric Hematology and Oncology. 2014 May; 31(4):327-39.

III. **Mattsson K***, Honkaniemi E*, Barbany G. & Gustafsson B. Increased p53 protein expression as a potential predictor of early relapse after hematopoietic stem cell transplantation in children with acute myelogenous leukemia. *Shared first authorship. Pediatr. Transplant. 2015 October; 19, 767–775.

IV. **Mattsson K,** Honkaniemi E, Ramme K, Barbany G, Sander B and Gustafsson B. Strong expression of p53 protein in bone marrow samples after hematopoiteic stem cell transplantation indicates risk of relapse in pediatric acute lymphoblastic leukemia patients. Submitted.

CONTENTS

LIST OF ABBREVIATIONS

ALL	Acute lymphoblastic leukemia
AML	Acute myeloid leukemia
BM	Bone marrow
CAR	Chimeric antigen receptor
CML	Chronic myeloid leukemia
CNS	Central nervous system
CR	Complete remission
DCL	Donor cell leukemia
DLI	Donor lymphocyte infusion
FFPE	Formalin-fixed parrafin embedded
GVHD	Graft versus host disease
GVL	Graft versus leukemia
HLA	Human leukocyte antigen
HSCT	Hematopoietic stem cell transplantation
IHC	Immunohistochemistry
JMML	Juvenile myelomonocytic leukemia
LAP	Leukemia associated phenotype
MDS	Myelodysplastic syndrome
MPFC	Multiparameter flow cytometry
MRD	Minimal residual disease
NBS	Neonatal blood spot
NOPHO	Nordic Society for Pediatric Hematology and Oncology
PCR	Polymerase chain reaction
RSS	Recombination signal sequences
TKI	Tyrosine kinase inhibitor
TMA	Tissue micro array
WBC	White blood cell
WGA	Whole genome amplification
WHO	World Health Organization

1 INTRODUCTION

1.1 LEUKEMIA

Cancer- an abnormal growth of cells which tend to proliferate in an uncontrolled way - is the name we use for over two hundred different diseases that differs in their tissue of origin and genetic composition. This thesis concerns pediatric leukemia, a cancer that arise from blood cells and that is the most common malignancy in children.

1.1.1 Short history of leukemia

In 1844 a French physician named Alfred Donné observed several patients with increased amount of white blood cells (WBCs). He suggested that it would be due to developmental arrest of the WBCs. One year later in 1845, the British professor John Hughes Bennet described the abnormalities he observed in the blood during an autopsy of a 28-year-old man, in a post mortem report. He called it "leukocythemia" and suggested that was due to a systemic hematopoietic disorder rather than caused by inflammation. After similar observations in an autopsy of a 50-year-old women later the same year, the German pathologist Rudolf Virchow published a paper with the title "Weisses Blut" meaning "White Blood". A few years later he named the disease "Leukämie" after the Greek words *leukos* meaning clear or white, and *haima* meaning blood [1].

In 1850, the first report of leukemia in a pediatric patient was published by Henry William Fuller who described a 9-year-old girl who had been admitted at St Georges Hospital in London with symptoms such as frequent bleedings, high white cell count in blood and an enlarged spleen. Fullers detailed description of the case has later allowed experts to believe that this was a case of chronic myeloid leukemia (CML) [2]. The girl died from her disease after just a few months and pediatric leukemia continued to be a fatal disease for many years.

One century later, in 1948 the use of chemotherapeutic substances showed to induce temporary remission in leukemic patients. In 1961, after treating 39 leukemic pediatric patients with a combination of two chemotherapeutic agents, Frei *et al* reported complete remission in 59%, and a 2-year survival rate in 20% of patients in the same group [3]. A year later at St. Jude Children's Research Hospital a new approach to cure leukemia was induced including multiple therapies, central nervous system (CNS) -directed therapy with cranial radiation, therapies including remission induction, consolidation therapy and continuation treatment [3]. This laid the foundation for modern therapy where multiple target therapy is still the backbone for treatment of leukemia.

Another milestone in leukemia treatment was in 1957 when E. Donnall Thomas published a paper in *New England Journal of Medicine* where he had treated six patients with infusion of bone marrow (BM) cells from an unrelated donor. Despite engraftment in two patients, all six patients died within 100 days [4]. Nevertheless the potential of the treatment was not forsaken, and since then the knowledge concerning patient specific human leukocyte antigens (HLA) has increased. Today, the patients can receive a hematopoietic stem cell transplantation (HSCT) from an HLA compatible sibling donor, as well as a matched unrelated donor. The donor graft is supposed to replace the patient graft and thereby induce a graft versus leukemia (GVL) effect. Other factors that contribute to a better overall survival after transplantation are e.g early detection and treatment of virus infections and good nutritional care. Today allogeneic stem cell transplantation is a lifesaving treatment for many leukemia patients.

Due to better knowledge concerning the biology and the genetics of the disease the treatment of leukemia has improved, with e.g optimized dosage of chemotherapy, more target directed drugs, early detection of infections and better supportive care. The 5-year survival rate for pediatric leukemia has increased from 10% in the 1960s to almost 80% in the 1990s [5]. Since then survival rates have continued to slowly rise and are now 90% for pediatric acute

lymphoblastic leukemia (ALL) cases and close to 70% for pediatric acute myeloid leukemia (AML) cases in several western countries [6,7].

Yet, there are still children who cannot be cured from their disease and hopefully more research will continue to improve the prognosis for all children diagnosed with leukemia.

1.1.2 Pediatric leukemias

Approximately 70 children are diagnosed with leukemia each year in Sweden [8]. Leukemia can be separated into several different subtypes, depending on their origin of the stem cell linage in the bone marrow; lymphoid or myeloid origin.

1.1.2.1 Acute lymphoblastic leukemias

ALL affects the lymphoid cells and accounts for approximately 80% of all pediatric leukemia cases. ALL can affect both B and T- cell linages and results in an increased amount of immature and non-functional lymphoblasts. For B-cell ALL there is an incidence peek between 2-5 years [9]. T-cell ALL is a rare form of pediatric ALL, accounting for about 15% of acute lymphatic leukemias. T-cell ALL is more prevalent in older children with a boy to girl ratio of 3:1. Long term survival rates in children with ALL have reached 90% in certain developed countries and for T-cell ALL they are 70-90% [6,10,11].

1.1.2.2 Myeloid leukemias

The myeloid leukemias are further divided into different subtypes. AML accounts for approximately 15% of all childhood leukemia cases. In turn, AML patients can be even further divided into different risk groups considering morphology, karyotype and molecular alterations, according to the World Health Organization (WHO) classification system. For children with AML survival rates are approaching 70% [7,12].

Approximately 5% of pediatric leukemias consist of the rare myeloid malignancies myelodysplastic syndrome (MDS), juvenile myelomonocytic leukemia (JMML) and CML [8,12].

MDS is a rare disease in children defined by clonal defects causing unsuccessful hematopoiesis and symptoms such as neutropenia and thrombocytopenia and predisposition to develop AML. Approximately 50% of children with MDS are cured by HSCT [13].

JMML is an aggressive proliferative disorder characterized by excessive proliferation of monocytic and granulocytic cells, mostly affecting young children and the event free survival after HSCT is around 50% [13,14].

CML mostly affects older children >10 years, often presented with splenomegaly and high WBC counts. CML is characterized by presence of the fusion gene *BCR-ABL,* also called the Philadelphia chromosome [15]. There are three clinical phases of CML; chronic, accelerated and blast crisis, and treatment success correlates to what phase the patient is diagnosed in. Most patients are diagnosed in chronic phase and long term survival rates are between 60-80% [16].

1.1.3 Treatment

Improvement in treatment such as better risk classification, improved therapies, better supportive care and discovery of clinical usable tools to monitor disease progression has resulted in significant enhanced survival in children with leukemia. Pediatric leukemias are treated according to different regional protocols often based on biological, clinical and genetic features such as lymphoid or myeloid origin, age of the patient, WBC levels, CNS status and genetic markers. In Sweden pediatric leukemia patients are treated according to protocols from the Nordic Society for Pediatric Hematology and Oncology (NOPHO). Response to therapy, measured through minimal residual disease (MRD) analysis, has emerged as an important factor for risk stratification during treatment. Choice of therapy is

based on current knowledge in the field, for example when tyrosine kinase inhibitors (TKIs) showed to induce remission in leukemia patients positive for the Philadelphia chromosome, the majority of this group of patients was no longer directly considered candidates for HSCT [17]. Yet, for AML patients, classified as high risk and in complete remission (CR) 1 or those with poor response to initial treatment, HSCT has shown to be a preferable therapy to cure disease [18,19]. In recent years genetically engineered T-cells expressing a chimeric antigen receptor (CAR) has shown great potential in treatment of pediatric ALL patients who relapsed or responded poorly to conventional therapy [20].

1.1.3.1 Relapse

Relapse is one of the major obstacles in treatment of leukemia. Studies have shown that the leukemic clone seen at relapse may differ from the clone identified at diagnosis. Sometimes the relapsed clone may have been present as a minor subclone when the leukemia first was diagnosed, in other cases the relapsed clone is identical to, or even very different from the original clone. The relapsed clone tend to be more resistant to chemotherapy which in one reason why patients who relapse have poor prognosis [17]

1.1.3.2 Hematopoietic stem cell transplantation

Although the general survival has improved remarkably in the last decades there is still a small portion of children with more aggressive malignancies that are candidates for HSCT such as patients with JMML and MDS [14]. Furthermore, ALL and AML patients diagnosed with specific cytogenetic or molecular markers indicating unfavorable prognosis, some children that relapse as well as children with poor response to chemotherapy, are also candidates for HSCT. For example, after induction chemotherapy 90% of children with ALL and AML achieve CR, but 10-15% of ALL cases and 30–40% of AML cases still relapse [12,21–24]. Patients that are detected with an intermediate response after first induction therapy, yet receive morphological remission after the second induction still have a high risk of relapse [24]. The over-all survival for ALL patients who relapse is poor, about 50% [25]. In this

group of patients, there is still a need for new clinical and biological markers that can indicate prognosis and thereby aid in the decision of what therapy to give [21,26].

Even though HSCT offers an effective and life-saving treatment, 20-60% of children still relapse after HSCT making it the major course of treatment failure in children that undergo HSCT due to malignant disease [27–30]. New prognostic markers indicating a forthcoming relapse after HSCT would be a major contributor to more individualized therapy for the vulnerable group of children with more aggressive leukemia.

1.1.3.3 Prognostic tools after HSCT

There are two major tools for monitoring success of treatment after HSCT; analysis of MRD and chimerism. In brief, MRD analysis measures the leukemic clone directly whereas chimerism analysis measurers the ratio of donor/recipient graft in the bone marrow [26]. An ideal MRD marker should be present in all leukemic cells and absent from all non-leukemic cells. A detected MRD or elevated recipient cells in the chimerism analysis demonstrate the need for therapeutic interventions such as lowering of immunosuppression or donor lymphocyte infusion (DLI). These interventions which will boost the GVL effect however, might induce graft versus host disease (GVHD) which is another severe and potential life-threatening condition, hence there is a delicate balance to consider when deciding what therapy to give these patients [31]. DLI was first shown to be effective in CML-patients after HSCT by Kolb *et al.* Administration of DLI has shown to induce CR in 8% of ALL-patients and 22% of AML-patients. However, if chemotherapy is used to reduce tumor burden before DLI CR is further improved to 33% and 37% in ALL and AML patients respectively [32]. DLI has also shown to be effective when given if mixed chimerism is detected without signs of MRD [31]. Therefore chimerism test and MRD is recommended as a regular schedule in the post-transplant period to improve the possibility of detecting a forthcoming relapse early [32,33].

1.1.3.4 Minimal residual disease

MRD is currently the most effective tool to monitor effectiveness of treatment in ALL and has also emerged as a reliable tool in AML [6,9]. MRD are used for risk stratification and can help to identify those patients who may benefit from a HSCT. Also, MRD levels before HSCT are strongly associated with outcome after HSCT [29,30,32].

Two different approaches are used to detect MRD, clone-specific quantitative polymerase chain reaction (PCR) and multiparameter flow cytometry (MPFC) detecting abnormal immunophenotypes, both reaching a sensitivity of 10^{-4} cells [34]. PCR is used to clone breakpoints of common leukemic fusion genes and genetic mutations previously identified in the leukemic cells. The usefulness of these aberrations as targets for MRD depends on their stability during the course of the disease. MPFC detects leukemia associated phenotypes (LAPs) as leukemic cells often present hematopoietic antigens, such as T-cell receptors and immune globulin gene rearrangements, in combinations that are unusual in healthy cells thus provide a possibility to monitor survival of the malignant cells after treatment [35]. A detected MRD of ≥0.01% leukemic cells at day 29 after induction therapy indicates the need for intensified treatment in patients with ALL. MRD is also used for risk assessment in AML patients where the generally accepted lower limit for detection is 0.1% of leukemic cells, and high MRD, despite morphological remission might indicate HSCT [24,36].

1.1.3.5 Chimerism

Chimerism, analyzed by PCR using short tandem repeats to identify donor and host graft, monitors the possible event of re-appearance of recipient cells after HSCT. Patients with rapid increased mixed chimerism after HSCT may be at risk of relapse [26,33].

1.1.4 Etiology

The etiology of most pediatric leukemias are unknown. Less than 5% of cases are believed to be due to germ-line genetic aberrations resulting in syndromes such as Down syndrome or

Bloom syndrome, or to ionizing radiation or exposure to chemotherapeutic drugs [37]. Many studies throughout the years have investigated different possible risk factors such as parental or early childhood exposure to non-ionizing electromagnetic fields, pollution, and maternal smoking during pregnancy however, none of these have been clearly implicated [38]. Old parental age has been associated with increased risk of developing childhood leukemia in the offspring in some studies [39,40]. Also, vaccination and virus-studies has been performed without conclusive results [38,41]. Children born with Li-Fraumeni syndrome, caused by inherited mutations in the *TP53* gene, have an increased risk of developing leukemia. Being a twin does not mean an overall increased risk of developing leukemia but if one of the twins develop disease during early childhood an increased risk has been observed for the second twin, which might indicate a possible pre-natal genetic involvement [38].

Two major hypothesis, both suggesting that infections promote pediatric leukemia, has been proposed in the literature; Kinlens "population mixing" theory and Greaves "delayed infection" theory. Kinlen suggested that the immigration of people to previously remote areas exposing the original inhabitant's immune defenses to previously un-encountered infectious agents could trigger the development of leukemia. Greaves proposed that leukemia development is generated by genetic aberrations occurring in a two-step matter where the first step is prenatal and the second step is post-natal and may be triggered by an infection [38,41]. The role of infections in ALL is still controversial [42]. However, there is a growing body of evidence indicating that the initial event is a genetic aberration occurring prenatally in children that later develop leukemia, as discussed further in section 1.3.

1.2 MOLECULAR MARKERS IN PEDIATRIC LEUKEMIAS

1.2.1 Acute lymphoblastic leukemia

Molecular markers have had a great implication in treatment of B-cell ALL, both in risk stratification and as targets for MRD analysis. Examples of cytogenetic markers commonly

used for risk stratification in ALL are *ETV6-RUNX1* (t(12;21)) and high hyperdiploidy (> 50 chromosomes) which occurs in about 25% of childhood ALLs respectively. These markers are associated with good prognosis and the overall survival for patients with *ETV6-RUNX1* positive ALL or a high hyperdiploid karyotype are over 93%. Markers indicating poor prognosis hence indications for HSCT are the fusion gene *BCR-ABL* (t(9;22)), translocations involving the *MLL* gene with different partner genes and, hypodiploidy (< 44 chromosomes) which are found in approximately 3%, 5% and 1% of cases respectively [17,43,44].

Frequency of translocations varies with age as *ETV6-RUNX1* and *BCR-ABL* are more common in older children whereas translocations involving the *MLL* gene are present in 80% of patients less than 1 year [9]. Among this subgroup of infant *MLL*-positive patients the 5-year survival is 50% [17].

Intrachromosomal amplification of chromosome 21 (iAMP21) is due to instability of chromosome 21 and occurs in about 2% of ALL patients, mostly in older children. It was previously associated with poor prognosis but can now be treated with intense chemotherapy [17]. Other chromosomal abnormalities such as dic(9;20) and t(1;19) occurs in approximately 2% and 3% of pediatric ALLs respectively, and children with these aberrations are classified as intermediate risk in the NOPHO protocol for treatment of pediatric ALL [45].

Deletions the *IKZF1* gene have been associated with poor prognosis, and are found in approximately 15-16% of B-cell ALLs [44,46–48]. It is often seen in patients with Philadelphia-positive ALL and associated with poor outcome despite TKI therapy suggesting that these patients could benefit from more intense treatment [46]. Germline variations of *IKZF1* have been found in both familial childhood ALL and sporadic ALL indicating that alterations in this gene may have a predisposing significance in pediatric ALL [49].

TCF3-HLF (t(17;19)) is another fusion gene that has been associated with very poor outcome. *TCF3-HLF* is rare and have mostly been identified in older children [50].

Deletions of the *CDKN2A* gene, encoding the protein p16, has been reported in 25-36% of pediatric B-cell ALL cases and associated with high WBC count and poor prognosis [44,46,51].

Although *TP53* mutations are rare at diagnosis of pediatric ALL, they are more common in certain subgroups such as hypodiploidy ALL or ALL with *N-MYC* rearrangements [52,53]. Mutations of *TP53* are associated with lower overall survival (OS), lower event free survival (EFS) and higher relapse rate (RR) [54]. Alterations in *TP53* in pediatric leukemia is discussed more in section 1.4

1.2.2 T-cell acute lymphoblastic leukemia

In contrast to B-ALL, and although chromosomal translocations are present in 50% of T-cell ALL their prognostic indications are not well identified and therefore not routinely used for risk stratification. *TLX3* rearrangements have been found in more than 20% of patients but the prognostic significance is not established [55,56]. The fusion gene *STIL-TAL1* is present in 11-27% of children with T-cell ALL at diagnosis [55,57–62]. *STIL-TAL1* is exclusively found in T-cell ALL and can be used as a tool to monitor MRD during treatment therapy [63], however, the prognostic value of this genetic aberration is not established although preliminary data show a favorable prognosis [55,56,59,60,62]. It has been suggested that this fusion gene could be an early or initiating event in T-cell ALL [64]. Despite these observations the fusion appears to be very rare at diagnosis in infant T-cell ALL patients [65], but its presence increases with age, peaking at 10-15 years [55,58]. Yet, *STIL-TAL1* it is still more common in children and adolescents than in adults [66]

NOTCH1 mutations are seen in more than 50% of T-cell ALL and may indicate a good prognosis [17,67]. Deletions in the *CDKN2A* gene are found in 60-80% of pediatric T-cell ALL patients [68]. Mutations in the tumor suppressor gene *PTEN* was found in 12% of 257 pediatric T-cell ALLs in a study that indicated that *PTEN* mutations could have inferior prognosis [67].

1.2.3 Acute myeloid leukemia

AML is a very heterogeneous disease with a great range of genetic aberrations yet the use of risk markers has not been as extensively used as in ALLs. However, molecular markers commonly seen in AML include *RUNX1-RUNX1T1* (t(8;21)), inv(16) and *MLL* rearrangements and mutations in *FLT3, NRAS, PTPN11* [12]. Mutations in *NPM1* and overexpression of *WT1* are also found in AML patients and are target candidates for MRD analysis [36,69]. *FLT3* , which is an indication for HSCT, and *WT1* are associated with poor outcome whereas *NPM1* and the *MLL* rearrangement t(1;11) is associated with a favorable outcome in AML patients [12].

1.2.4 Myelodysplastic syndrome

Monosomy 7 is the most frequent molecular marker in MDS. Germline mutations in *GATA2* and *RUNX1* are associated with predisposition to MDS [70]. Also, mutations in genes involved in the Ras-MAPK pathway such as *PTPN11* and *NRAS* are common in pediatric MDS patients [71].

1.2.5 Juvenile myelomonocytic leukemia

Monosomy 7 is also found in 25% of JMML cases. Somatic mutations in *KRAS, NRAS* and *PTPN11* are present in 50% of JMML patients [14]. *PTPN11* also occur as germline mutation in JMML patients with Noonan syndrome [13]. Several of the genetic alterations found in JMML patients are involved in the RAS signaling pathway yet they are associated with different prognostic outcomes as *NRAS* indicates good prognosis and *PTPN11* seems to have an unfavorable outcome [13,14].

1.2.6 Chronic myeloid leukemia

Chronic myeloid leukemia is characterized by the *BCR-ABL* fusion gene [72]. The fusion induces a constantly activated tyrosine kinase which results in increased cell proliferation.

The altered tyrosine kinase activity can be inhibited by TKIs, which can result in long term remission in most patients [16].

1.3 EVIDENCE FOR PRENATAL ORIGIN OF PEDIATRIC LEUKEMIA

Chromosome translocations could be the first events in leukemogenesis, occurring *in utero* in some cases of pediatric leukemia [73]. These genetic changes are considered to be necessary, but not sufficient to cause overt leukemia. Studies of NBSs confirm that some leukemias may have their origin *in utero*, when myeloid and lymphoid cells are not fully differentiated and are therefore more sensitive for malignant transformation [73]. Several important factors affecting the potential prenatal phase of ALL are unknown, such as time of initiation and causes of initiating events [74]. Hence genetic alterations could originate at time of conception or later during hematopoiesis *in utero*.

Foetal haematopoiesis begins in the haematogeneic endothelium from the yolk sac, then mainly continues in the liver and is finally established in the bone marrow and spleen [75]. The yolk sac is the major site of embryonic erythropoiesis with the primary purpose to produce red blood cells that can facilitate tissue oxygenation as the embryo undergoes rapid growth. These erythroid progenitor cells are not pluripotent and do not have renewal capability. Stem cell development occurs later and give rise to all blood lineages. These migrate to the fetal liver and then to the bone marrow, which is the final location for hematopoietic stem cells [76]. The B-lymphocytes arise in the liver at 8 weeks of gestation but can be detected in the blood circulation at gestational age of 12 weeks. T-cells progenitors, also derived from the liver from seven weeks of gestation, migrate to the thymus by 8 - 9 weeks of gestation and circulating mature T-lymphocytes are first detected around gestational week 15 to 16 [77].

Through studies of NBSs and monozygotic twins some childhood ALLs can be traced back to a prenatal origin, starting *in utero* with a preleukemic clone, followed by the acquisition of postnatal genomic losses or gains subsequent to the prenatal primary cytogenetic aberration

[65,78–84]. Genetic aberrations that have been traced back to a prenatal origin are *MLL-AF4*, *ETV6-RUNX1*, *ETV6-ABL1* and *BCR-ABL* fusions [78,82,85–87].

Ford *et al*, were first to identify the same unique fusion sequence for *ETV6-RUNX1* in a pair of monozygotic twins who developed leukemia at the ages of three and four years respectively. The authors suggested that this genetic aberration was not sufficient for leukemia development, hence secondary alterations are required for disease to occur [88]. This was further supported in a study by Ma *et al*, where identical breakpoints for the fusion gene *ETV6-RUNX1* were identified in a pair of monozygotic twins in bone marrow sampled at the time of diagnosis of ALL, also indicating a *in utero* origin for the translocation [81]. They also found secondary genetic alterations that were not identical between the twins and that likely occurred after birth. This indicates that *ETV6-RUNX1* may be an early event in leukemia development however, secondary events are necessary for ALL to arise [81,89]. Further support for the need of secondary post-natal mutations are found in a study where the *ETV6-RUNX1* fusion RNA was detected in cord blood in 1% of 567 healthy individuals, which is a higher prevalence than the occurrence of ALL in children [90]. These results were also supported by Zuna *et al*, who detected the *ETV6-RUNX1* fusion in 2% of NBS from 256 healthy newborns [91]. However, a larger study found no evidence of *ETV6-RUNX1* transcripts in cord blood from 1417 healthy individuals, and another study could not detect the *ETV6-RUNX1* transcript in RNA extracted from NBSs from fifteen children who later developed leukemia or in thirty controls [92,93]. Nevertheless, in a most recent study the *ETV6-RUNX1* was detected in 5% of newborns and the authors suggest that the controversy regarding the prevalence of this fusion gene in healthy newborns may be due to differences in the methods used [94]. Taken together these findings suggest that the genetic event giving raise to the *ETV6-RUNX1* fusion gene is a primary event that occurs in utero, however it has a very low potential of inducing leukemia.

In a study by Cazzaniga *et al*, *BCR-ABL1* fusion genes were detected in two twin pairs. One pair, both diagnosed with ALL shared the genomic sequence for the fusion gene, and it was also traced back to their NBSs, indicating that it was a pre-natal event. The other pair both expressed *BCR-ABL1* positivity in their NBSs however, only one twin developed leukemia. This second finding also demonstrates that secondary post-natal mutations are needed to develop disease [82].

Evidence for an *in utero* origin for *MLL* rearrangements and a hyperdiploid karyotype in pediatric leukemia has also been proposed in the literature, indicating that also these aberrations may be of importance as first events in leukemogenesis [78,95–97]. Also, *ETV6-ABL1* has been traced back to the NBS from a boy that developed B-cell ALL at the age of two [87].

Recognition of a strong association between preleukeimc fusion genes and development of disease could also encourage screening of donor source prior to HSCT. Donor cell leukemia (DCL) have been reported to represent 5% of leukemic relapses after HSCT [98]. Although DCL is very rare, it is a severe event with poor outcome [99,100]. It has been suggested that DCL occurrence is similar amongst the different sources of donor cells, such as bone marrow, peripheral blood stem cells and cord blood. [101].

1.4 *TP53* AND PEDIATRIC LEUKEMIA

TP53 is a tumor suppressor gene located at the short arm of chromosome 17. The *TP53* gene encodes the p53 transcription factor protein p53, which has a key role in regulating the cell cycle by preventing damaged DNA to be replicated. Normally p53 protein levels are very low in a cell. p53 is regulated by another protein MDM2, which ubiquiylates p53 and thereby targets it for degradation. However, in responds to several stress factors such as DNA damage, hypoxia and oncogene activation p53 gets phosphorylated and is no longer recognized by MDM2 and levels of p53 rises in the cell. p53 acts by initiating transcription of genes such as p21 which in turn inhibits Cdk2 and thereby causes cell cycle arrest, and

PUMA and BAX that by activating different signaling pathways initiate apoptosis. Because of its important role in preventing damaged DNA to replicate, p53 is often called the "guardian of the genome" [102]. *TP53* alterations are reported with a frequency of 10-40% in different adult hematological malignancies, mainly seen in disease with a progressive course [103,104]. The knowledge regarding prevalence and impact of *TP53* alterations in pediatric hematological malignancies is also increasing.

In pediatric ALL, alterations of *TP53* appear to be rare at diagnosis yet more common in relapsed cases. *TP53* mutations have been found in 2-3% of children at diagnosis of ALL, but in 10-12% after relapse, indicating involvement in progression to more aggressive disease [43,54,105–109]. Yet, one study found *TP53* mutations in 14.5% of sixty-two children with ALL and the patients with *TP53* mutations also had a high expression of p53 however, these results were not associated with outcome [110]. Gustafsson *et al,* have previously reported an increased expression of p53 protein in bone marrow samples from a group of children eligible for HSCT diagnosed with ALL, AML, MDS and CML, compared to a group of patients with non-malignant bone marrow disorders [111]

For the myeloid malignancies the data is less conclusive. One study found *TP53* mutations in 1.1% of 206 pediatric AML patients and, the patients with *TP53* mutations had poor outcomes [112]. Immunohistochemical analysis of a mutant form of p53 expression was suggested as a possible method for detecting MRD in a case report of a pediatric patient with AML [113]. In another study, no *TP53* mutations were detected in twenty-seven patients with AML or in two patients with CML [106]. In a study analyzing *TP53* mutations in twenty JMML patients, and also p53 expression in eight of these patients, results were negative [114]. Saito *et al*, also analyzed *TP53* mutations in eighteen patients with JMML with negative results. However, in the same study they found a *TP53* mutation in one out of nine patients with MDS and this patient also had an overexpression of p53 in 20% of BM cells and a poor outcome [115]. Silveira *et al,* found loss of *TP53* in 95% of patients in a study group consisting

of nineteen children with MDS, suggesting that this alteration may be involved in development of pediatric MDS, whereas Schwartz *et al*, only found *TP53* mutations in 4% of seventy-seven children with MDS [71,116]. Contrary, in a study investigating the mutational differences in adult and pediatric MDS, no mutations in the *TP53* gene were detected in 50 patients below 18 years of age, and the authors suggested that this mutation is not involved in pathogenesis of pediatric MDS, which were in agreement with the results and conclusion from yet another study where no *TP53* mutations were found in 35 children with MDS [70,117].

1.5 P16, P21 AND PTEN

Mutations in the *CDKN2A* gene, encoding protein p16, are common genetic alterations in cancer and are found in approximately 30% of pediatric ALL cases and in 60-80% of T-cell ALL cases [44,51,68]. In the cell cycle p16 inhibits the cyclin-dependent kinase 4/Cyclin D complex therefore induce phosphorylation of the retinoblastoma protein RB1 and promote cell cycle progression.

p21 protein, the product of the *CDKN1A* gene, is regulated downstream of the p53 protein and activation of this protein induces cell cycle arrest [118]. Alterations in the p21 pathway has been correlated to leukemogenesis in *AML1-ETO* positive AML [119]. In adult patients with MDS, low levels of p21 has been associated with progression to AML and lower overall survival rates [120].

PTEN (phosphatase and tensin homolog) is a tumor suppressor gene and one of the most altered genes in cancer with mutations seen in 30-80% of different cancers such as endometrial carcinoma, glioblastoma, prostate, breast, colon and lung cancer. Germline mutation in this gene causes Cowden Disease and predisposition to several cancers [121]. Deletions of PTEN has also been found in approximately 10% of pediatric T-cell ALL [67,122].

2 AIMS OF THE THESIS

The general aim of this thesis was to increase our understanding regarding the origin and development of pediatric leukemia. This was done by investigating if a specific leukemia associated genetic aberration can be traced back to a prenatal origin (*study I*) and, by evaluating if protein expressions differs between leukemic patients during treatment, hence can be predictive for relapse after hematopoietic stem cell transplantation in children with leukemia (*study II-IV*). Specific aims were;

Study I

Is the fusion gene *STIL-TAL1* present in DNA from neonatal blood spots from thirty-eight pediatric patients with T-cell ALL?

Study II

Can aberrant expression of the cell cycle regulating protein p53, analyzed by immunohistochemistry (IHC), act as a predictive marker for relapse after HSCT in children with MDS, JMML and CML? Is altered p53 expression due to underlying mutations in the *TP53* gene?

Study III

Does altered expressions of the cell cycle regulating proteins, p53, p21, p16 and PTEN, analyzed by immunohistochemistry, have potential as predictive markers for relapse after HSCT in children with AML?

Study IV

Can expression of the cell cycle regulating protein, p53, analyzed by immunohistochemistry, act as a predictive marker for relapse after HSCT in children with ALL?

3 MATERIAL AND METHODS

3.1 STUDY I

3.1.1 Neonatal blood spots

In Sweden, blood spots from newborns have been taken within 48 hours after birth since the mid-60s, with the main purpose to analyze for twenty-four rare but often treatable diseases, such as endocrine and metabolic disorders. The NBSs are collected onto filter paper called Guthrie Cards. Since 1975, Guthrie cards have been saved and stored for the main purpose to be used in quality controls of screening program itself and for potential future use in medical care. However, this unique material can also be used for research after ethical approval.

Previously, Guthrie cards from 417 Swedish children who developed childhood B-cell or T-cell ALL in 1992-2006, together with 834 controls matched by birth date and birth place, have been collected. From each Guthrie card four small spots (3 mm in diameter) were punched out, estimated to contain approximately 12 µl of blood corresponding approximately 180,000 leukocytes and 120,000 lymphocytes. The median storage time for the NBSs was 9.9 years (range 2.6 - 22.8). DNA extraction was carried out by the minimal essential medium (MEM) method, based on a method described by Barbi *et al*, 1996 and modified according to Priftakis *et al*, 2003 [123,124]. The DNA yield was ascertained by analyzing the samples for the human albumin gene by TaqMan real-time PCR. The DNA obtained has previously been used in several studies, where the primary aim was to find out if particular viruses could be involved in leukemia development, but no association between in utero infection by herpes virus, adenovirus or polyomavirus and development leukemia could be established [125–127].

For this thesis, I had the opportunity to use some of the frozen NBS DNA to investigate if the fusion gene *STIL-TAL1*, common at diagnosis of T-cell ALL, could be traced back to early stages of life in thirty-eight children who developed T-cell ALL between the ages of 1 and 17 years.

3.1.2 *STIL-TAL1* fusion gene

The *TAL1* (T-cell acute leukemia) gene, located at 1p23, consists of eight exons, while the *STIL* (SCL/TAL1 Interrupting Locus) gene located at 1p32 consists of eighteen exons. *STIL-TAL1* gene fusion occurs after an approximately 90kb interstitial deletion on chromosome 1, which joins *TAL1* to the *STIL* gene and results in inappropriate activation of the TAL1 protein through the *STIL* gene promoter. Several deletions resulting in *STIL-TAL1* gene fusion have been described yet two of them, type I and type II, appear to be most common and represent 95% of total *STIL-TAL1* cases and occur in 10-19% and 1-3% of pediatric T-cell ALL patients respectively [57,58,62]. Type I and II deletions appear to be driven by aberrant RAG recombinase activity since the breakpoints usually cluster at heptamer-nonamer recombination signal sequences (RSS); hallmarks of V(D)J gene joining. Both type I and II deletions use the same cryptic heptamer RSS at the 5' break in the *STIL* gene but can use either one of two 3' RSS (only 1.7kb apart) found in the 5' region of *TAL1*.

3.1.3 Analysis of *STIL-TAL1*

To ensure material quantity, NBS DNA was whole genome amplified (WGA) using the standard protocol for REPLI-g Mini Kit from QIAGEN. The *NRAS* gene was used as an integrity control for presence of DNA in the WGA NBS material.

STIL-TAL1 type I and II fusions were analyzed independently by nested PCR based on a protocol described by Breit *et al* 1993 [57]. *STIL-TAL1* PCR was performed using the

Accuprime Supermix II kit from Invitrogen, Life Technologies. In the first PCR 2-4 µl (depending on the intensity of the integrity control in the gel electrophoresis) of amplified DNA was used with the original *STIL* and *TAL1* I/II primers, cycling conditions 3 min at 94°C 2 min at 60°C 3 min at 72°C for 40 cycles, 7 min 72°C then hold at 12°C. In the second PCR, 2-4 µl of first PCR product was used with secondary *STIL* and secondary *TAL1* I/II primers for (for primer sequences, see *Study I* -supplementary data), cycling conditions 1 min 94°C then 30 sec 94°C, 30 sec 60°C, 30 sec 72°C for 35 cycles with a final 5 minute extension at 72°C. PCR was analyzed by electrophoresis in a 2.5% agarose gel stained with ethidium bromide.

3.2 STUDY II-IV

3.2.1 Material and study population

Analysis of bone marrow is a standard procedure at diagnosis of leukemia and during treatment to monitor the patient's response to therapy. To preserve the material for morphology and IHC analysis bone marrow samples are formalin-fixed, and paraffin embedded (FFPE) which also allows long time preservation hence this material can then be used for reevaluations and also research.

We have collected FFPE BM samples from 133 children diagnosed with leukemia, who were treated with HSCT at Karolinska University Hospital 1997-2010. We collected material from diagnosis, time of HSCT and from routine controls at 3, 6, 12 and 24 months after HSCT. We also collected FFPE BM samples from 55 control patients, who had been investigated for bone marrow disorder but cleared from malignancy. In total, we collected 438 bone marrow samples.

The study group consisted of; forty-nine patients with B-cell ALL, seventeen patients with T-cell ALL, thirty-four patients with AML, nineteen patients with MDS, nine patients with

JMML and five children with CML. The control group consisted of 2 healthy bone marrow donors and 53 children with the following conditions; 4 children diagnosed with thalassemia, 13 children with aplastic anemia, 7 children with Fanconi aplastic anemia, 3 children with amegakaryocytic thrombocytopenia, 1 child with Mb Hurler, 1 child with severe combined immunodeficiency, 4 children with Kostmann disease, 2 children with Wiscott Aldrich syndrome, 8 children with idiopathic thrombocytopenia, 1 child with chronic granulomatous disease, 1 child with hemolytic anemia, 5 children with suspected malignant disease, due to pancytopenia, but no malignant disease discovered, 1 child with neutropenia and 2 children with glycogenosis (*Study II and III*). In a later assessment we decided to exclude conditions which are considered to be pre-malignant, hence children with Fanconi aplastic anemia, Kostmann disease and Wiscott Aldrich syndrome were excluded, leaving forty-two children composing the control group in *study IV*.

3.2.2 Tissue micro array, immunohistochemistry and Sanger sequencing

Tissue micro array (TMA) is a cost effective and material saving technique. In TMA, only a small quantity of tissue is used from each BM sample and one microscope glass-slide can fit 120 samples. For our studies, TMA was prepared at the Center for Molecular Pathology at Malmö University Hospital.

Two stances, one mm in diameter were punched out from each FFPE BM sample and placed in a paraffin block fitting 120 cores. The block was then cut on a microtome and the 4mm thick slices were placed on object glass and deparaffinized. Each block produced several comparable microscope glass slides that could be stained for the proteins of interest.

Immunohistochemistry was performed according to standardized protocols at the department of pathology, Karolinska University Hospital-Huddinge, using a Leica BOND-III machine. Diaminobenzidine-peroxidase reaction weakly counterstained with hematoxylin visualized the antibodies. A positive control on a separate glass was included

in each set of staining. Cells negative for the antibody in each sample served as negative controls.

Expression of p53, p21, p16 and PTEN protein was analyzed in a microscope at high resolution (40X). p53 was analyzed as both total expression meaning all cells positive for p53, and strong expression meaning cells that had an intense expression of p53 identified as dark brown color in the nucleus after IHC. Analysis was blinded hence me and my colleague, who analyzed the TMAs, did not know if the sample belonged to a patient or control. For inclusion in our studies, a minimum of 100 cells were to be counted from each patient.

For *study II*, Sanger sequencing was preformed according to standard protocols at the Department of Molecular Medicine and Surgery, Karolinska Institutet, Stockholm. M13-tagged primers were used for mutation analysis of exon 2-11 in the *TP53* gene.

3.2.3 Statistical analysis

All data was analyzed in Statsoft Statistica 11 (*study II*), 12 (*study III*) and 13.2 (*study IV*) and Excel 2011-2013.

Logistic regression, which is a predictive analysis used when the dependent variable (in our case *relapse*) is binary (yes/no), was used to analyze the value of the protein expressions as predictive markers for relapse after HSCT.

Box-plots were used in *study II* and *III* to visualize protein expression in the relapse group, the non-relapse group, and the controls at diagnosis.

In *study II*, a time-to event curve (Kaplan-Meier analysis) was performed were the two groups of patients compared were based on median number of cells with positive p53 expression at diagnosis (13.6%). A Log Rank test was preformed to analyze statistical

difference between the groups. Correlation between p53 and p21 expression was analyzed with a Spearman's rank-order correlation test.

Independent sample *t*-test were preformed to evaluate differences in p53 expression between the controls and the leukemic patients (*study II* and *IV*), and between the relapse group and the non-relapse group at all time points (*study III*).

Results were considered significant if $p < 0.05$.

4 RESULTS AND DISCUSSION

4.1 STUDY I

DNA from neonatal blood spots from thirty-eight children who developed T-cell ALL before the age of 18 were analyzed by nested PCR. *STIL-TAL1* fusion type I or II were not detected in any of the thirty-eight NBS samples. The *NRAS* control gene was detected in all WGA NBS samples confirming the presence of enough DNA in the samples. The control samples were positive for *STIL-TAL1* type I showing functional primers and PCR cycling conditions.

Negative results of NBS analysis cannot be defined as true. Although it is possible that the investigated aberration does not have a prenatal origin, it may be that the leukemic clone, if present in the NBS, was missed when punching out the four pieces of the NBS that was used in this study or had not amplified sufficiently in the peripheral blood to be present in the spot. Our material contained approximately 120 000 lymphocytes per sample. Another limitation with our study was that we did not know if any of the patients included in this study had a *STIL-TAL1* fusion gene at diagnosis. Yet, there are several reasons why our results could possibly represent true negative findings.

Approximately 11-27% of children with T-cell ALL present with the genetic aberration *STIL-TAL1* at diagnosis [55,57–62]. We calculated that, according to what is reported in the literature 4-10 children of our thirty-eight included patients could be assumed to have this genetic alteration. Patients with *STIL-TAL1* fusion often present with a high level of WBCs at time of diagnosis, and an higher prevalence of this fusion gene has been reported in males compared to females [55,59,60,62]. Due to the gender composition with a male to female ratio of 5:1 and the recorded high WBC count ($>100 \times 10^9$/l) in 18 of the 38 patients in our

study group, we still argue that this was a suitable group for analysis of *STIL-TAL1* fusion, hence valid use of this unique material and of research money.

4.2 STUDY II-IV

p53 expression was found to be aberrant in patients who relapsed after HSCT in all three studies but at different time points during treatment (Table 1).

Table 1: Characteristics and results of study II, III and IV

Study	Leukemic subtype	Number of patients	Time point for result	Results*
II	MDS, JMML and CML	33	Diagnosis	Increased p53 expression predicted relapse, OR 1.19 (95% CI: 1.02-1.40, p = .028)
III	AML	34	3-6 months post HSCT	Significant difference in p53 expression between relapse group and non-relapse group, with higher expression in relapse group (p = .010)
IV	ALL	46	0-3 months post HSCT	Strong p53 expression predicted relapse, OR 2.63 (95% CI: 1.08-6.40, p = .033)

*Logistic regression *study II* and *IV*. Independent t-test in *study III*.

Saft *et al*, showed that analysis of p53 expression by IHC is a clinical useful tool for prognosis in patients diagnosed with MDS. Strong expression of p53 in ≥1% of cells, measured by IHC and laser micro dissection was associated with transformation to AML and, to poor overall survival in a group of MDS otherwise classified as low- or intermediated risk patients [128]. Interestingly, in the same study they also found an underlying mutation in *TP53* in the cells that strongly overexpressed the protein. In our studies of myeloid malignancies (*study II* and *III*) we calculated and suggested cut off levels of approximately 20% (19% and 21.3% respectively) of p53 positive cells in children diagnosed with MDS, JMML, CML and AML. In contrast to the study by Saft *et al*, we considered all p53 positive cells which could

be an explanation to the large difference in cut off levels. However, in our *study IV* where we analyzed strong expression in ALLs, we suggest that > 2% of cells with strong expression of p53 indicated increased risk of relapse which is more in agreement with the results of Saft *et al*, indicating that intense p53 expression in only a few cells can be a signal of disease progression.

Furthermore, at time of diagnosis p53 expression was increased in patients with hematological malignancies as compared to non-malignant controls in all three leukemic groups studied, indicating an involvement in leukemia (Figure 1). The control group had a lower expression of p53 protein at diagnosis than the leukemic patients, even when children with pre-malignant disorders were included (see *study II* and *III*). The decision to exclude the pre-malignant disorders from the control group was made solely with the intention to refine our material and did not have any substantial impact on our results.

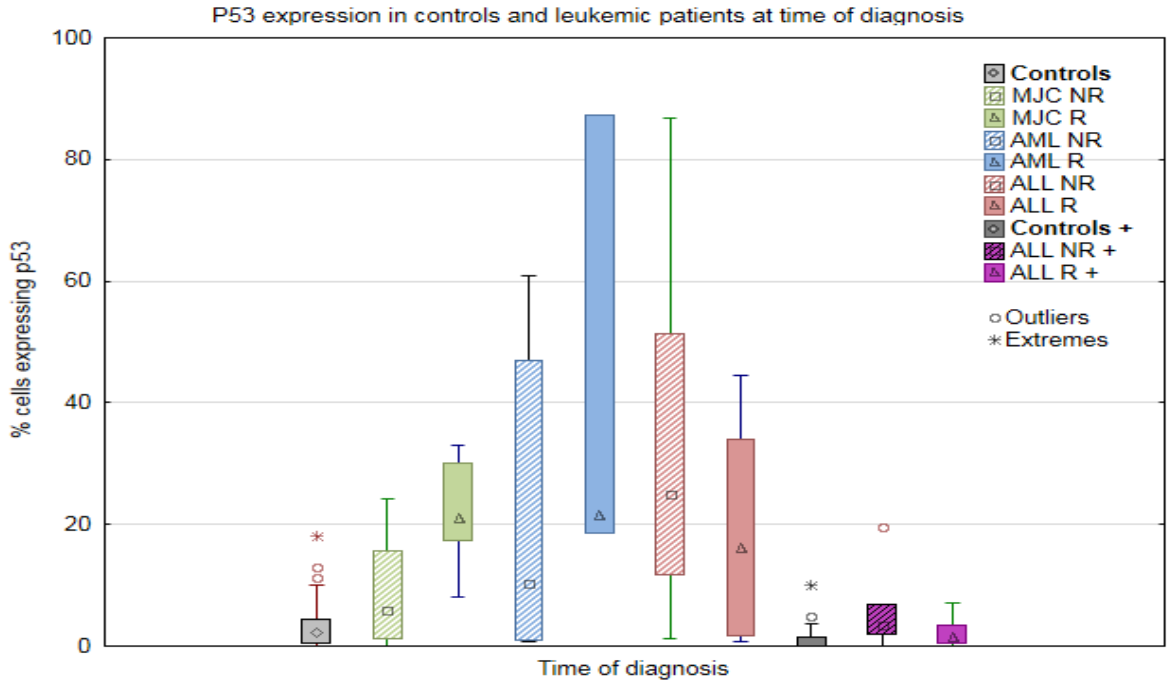

Figure 1: p53 expression at diagnosis in control group (n=42) compared to leukemic patients.

MJC = MDS, JMML, CML group, NR = non-relapse group, R = relapse group, + = strong expression.

An interesting observation at time of diagnosis is that in the myeloid malignancies (MDS, JMML, CML and AML) the group of patients who relapsed after HSCT have a higher expression of p53 at diagnosis compared to the non-relapse patients. However, at time of diagnosis for ALL patients, the non-relapse patients had a higher expression of both total and strong p53 than the relapse group. This indicates that the underlying mechanism for the increased protein expression differs between the malignant cells depending on their lineage.

In the group with MDS, JMML and CML patients, the significant result in our *study II* was seen at diagnosis which is in agreement with the findings in the paper by Jädersten *et al*, where *TP53* mutations were shown to be an early event in MDS patients [129]. It could be that the increased expression of p53 in the myeloid malignancies indicates a more aggressive disease as seen in MDS patients in previous studies [128,129]. In contrast to MDS, *TP53* mutations are rare at diagnosis of ALL and the increased percentage of cells expressing p53 seen in the non-relapse group could be a normal response to DNA damage or cellular stress as an attempt to prevent the tumor to proliferate. Yet, in some ALL patients the altered p53 expression may still represent a more aggressive clone that resists therapy and still remains after HSCT and thus may contribute to relapse. It would be interesting to study the underlying mechanisms of the altered p53 expression in the different leukemic subtypes to better understand the differences seen in our studies.

MRD analysis is important for accurate risk stratification and there are defined time points for these analysis in treatment protocols. These set times are sometimes delayed due to poor cellular bone marrow regeneration causing inadequate samples for laboratory analysis, hence bone marrow aspirations are postponed or repeated at a later time. It has been shown that delayed bone marrow aspiration for MRD analysis has resulted in lower levels of MRD hence this could affect risk stratification and in turn survival [130]. Another important factor for accurate MRD analysis are the numbers of markers that can be used in the analysis. One marker is associated with higher rate of negative results than two or more markers, as DNA

yield may be small in these patient samples the chances of successfully identify the same marker in two consecutive samples are limited. [130]. We have shown in our studies, that p53 expression is altered in pediatric ALL and AML patients after HSCT. If these alterations are due to an underlying genetic mutation in *TP53* in the leukemic clone, *TP53* analysis may have potential as a MRD marker in pediatric patients with leukemia after HSCT.

Although leukemia is the most common cancer in childhood it is still a rare disease. And as knowledge increases more leukemic subtypes arise, creating the possibility to more individualized treatment. It also encourages international research collaborations to attain homogenic study populations large enough to preform statistical analysis that one can draw valid conclusions from. One limitation in this thesis, especially for study II- IV, was the small sample sizes. To validate the potential of p53 analysis as a marker for relapse, one would like to take into account confounding factors such as; related or unrelated donor, donor source, donor age, conditioning, DLI and other factors that are known in influence outcome. This was not possible in our material due to limitations in the statistical methods when including too few participants.

5 CONCLUSION AND FUTURE PERSPECTIVES

Molecular markers are important tools to improve treatment and to achieve increased survival in children with leukemia. They can be used for risk stratification, monitoring disease progression and remission status, as target for development of new drugs, and for our essential need for an increased understanding of development of pediatric leukemia.

This thesis resulted in the following conclusions:

Study I: We suggest that *STIL-TAL1* fusion gene most probably occurs as a post-natal genetic event in T-cell leukemia.

Study II-IV: Aberrant expression of p53 protein seems to have potential as a predictive marker for relapse in different subtypes of pediatric leukemia. We conclude that increased p53 expression at diagnosis of MDS, JMML and CML may have potential as a marker to identify patients with inferior outcome after HSCT. For children with ALL and AML, monitoring of p53 expression after HSCT could be useful as a predictive marker for relapse in combination with conventional MRD and chimerism analysis.

Cancer therapies are severe and potential life-threatening treatments and it is vital that each patient´s treatment is in proportion to the severity and complexity of their individual disease. The importance of biological makers as an instrument to optimize risk stratification and as targets for new drug development in leukemia is repeatedly called for in the scientific literature.

Future studies would be necessary to validate the findings of our protein studies. They would need to include more patients; hence international collaborations should be encouraged. It would be interesting to explore the underlying mechanisms of the altered protein expression. One method that we have considered is single cell analysis, which makes is possible to pick out, and perform genetic analysis in the specific cells one would be interested in to investigate

further. This would be especially intriguing considering the results in our *study IV*, where an intense expression of p53 protein in approximately 2% of the cells was predictive of relapse after HSCT. I would also suggest that expression of the p53 regulating protein MDM2 and perhaps other p53 associated proteins were analyzed to indicate other possible underlying factors that can be the reason for the accumulation of p53 protein in the nucleus and that may affect the p53 pathway in the cell, as p53 is part of a complex cellular network [131].

6 POPULÄRVETENSKAPLIG SAMMANFATTNING

Leukemi drabbar ca 70 barn varje år i Sverige och utgör en tredjedel av all cancer hos barn. Den vanligaste formen av barnleukemi kallas akut lymfatisk leukemi (ALL), följt av akut myeloisk leukemi (AML) och tillsammans utgör dom ca 95% av barnleukemier (80% och 15% respektive). De mindre vanliga leukemidiagnoserna är kronisk myeloisk leukemi (KML), juvenil myelomonocytleukemi (JMML) och myelodysplastiskt syndrom (MDS). Sedan 1960-talet har barn som blir botade från leukemi ökat från ca 10% till idag då ca 90% av ALL patienter och nästa 80% av AML patienter överlever sin diagnos. För övriga diagnoser ligger överlevanden på ca 50-80%. Den positiva utvecklingen beror på de senaste decenniernas förbättrade behandling med mer specifika mediciner, bättre omvårdnad och mer kunskap om biologiska markörer som kan bidra till risklassificering av patienter, vara mål för medicinutveckling och som kan användas för att kunna följa eventuellt kvarvarande leukemiceller i barnet under och efter behandling.

Trots att de flesta barn med leukemi numer blir botade så finns det fortfarande en liten grupp som utvecklar en mer aggressiv sjukdom som kräver tuffare behandling och som då kan behöva genomgå en hematopoietisk stamcellstransplantation. Det gäller framför allt ALL och AML patienter som svarat dåligt på standardbehandling, de som fått återfall eller om de blivit diagnosticerade med specifika genetiska avvikelser. Alla barn med MDS och JMML får också genomgå stamcellstransplantation.

Vissa genetiska avvikelser är vanligt förekommande vid leukemidiagnos och intressant nog så har några av dessa kunna spårats tillbaka till barnets födelse genom studier av så kallade PKU-blodprov (nyföddhetsblodprov) där man kunnat påvisa att den genetiska avvikelsen fanns i några av barnets celler innan det insjuknade i leukemi.

Denna avhandling har fokuserat på biologiska markörer i barnleukemi då vi har tittat om vi kunde påvisa en genetisk avvikelse, som är vanlig hos barn som drabbas av en ovanlig form av ALL, i deras PKU-korts samt studerat om uttryck av specifika proteiner kan förutse ett

återfall hos barn som genomgått HSCT.

Studie I

STIL-TAL1 är en fusionsgen som förkommer hos ca 11-27% av alla barn som diagnosticeras med T-cells ALL. T-cells ALL utgör ca 15% av alla lymfatiska leukemier och är vanligare bland lite äldre barn runt 10 år. Vi undersökte förekomsten av denna fusionsgen i PKU-blodprov från 38 patienter som fått diagnosen T-cells ALL under barndomen. Genom att extrahera DNA från de sparade korten kunde vi med hjälp av specifika primersekvenser som fäster just på *STIL-TAL1* fusionsgenen kopierar upp den i en polymeraskedjereaktion och därmed undersöka om den gick att hitta i något av barnens PKU-kort. Vi kunde inte påvisa att något av barnen skulle vara fött med denna fusionsgen och vår slutsats är att denna genetiska avvikelse sker i ett senare stadie av leukemiutvecklingen.

Studie II-IV

Tumörsuppressorgenen *TP53,* är också är känd som "guardian of the genome" då den har en nyckelroll i celldelning. *TP53* kodar ett protein som ser till att skadat DNA inte förs vidare till en ny cell, genom att signalera lagning av DNA eller genom att initiera cellens självmordsprogram, kallat apoptos. *TP53* är den mest muterade genen i cancer. Ca 50% av solida tumörer har mutationer i denna gen och den har även hittats i ca 10-20% av hematologiska tumörer hos vuxna, vanligtvis förknippat med mer aggressiv sjukdom.
I ALL hos barn förekommer mutationer i *TP53* oftare hos de barn som får återfall i sin sjukdom efter initial behandling. Därför ville vi undersöka om ett förändrat uttryck av p53 proteinet kan förutse ett kommande återfall hos barn med mer svårbehandlad leukemi som får genomgå HSCT. p53 proteinet analyserades med immunhistokemi, en metod där specifika proteiner färgas och blir synliga i ett mikroskop. Vi undersökte uttrycket av p53 vid i de olika subgrupperna av leukemi vid diagnos, innan HSCT och vid efterföljande

rutinkontroller vid ca 0-3, 3-6 och 6-12 månader efter HSCT. I gruppen med 33 patienter med de ovanligare myeloida diagnoserna MDS, JMML och KML så var ett ökat uttryck av p53 vid diagnos predicerande för återfall efter HSCT. I gruppen med 34 AML patienter såg vi att de barn som fick återfall hade ett högre uttryck av p53 vid 3-6 månader efter HSCT än de som inte fick återfall. Slutligen analyserade vi 46 ALL patienter och då var det de som hade ett strakt uttryck av p53 vid 0-3 månader efter HSCT, dvs de som hade celler som var starkt färgade när man tittade i mikroskopet, som hade högre risk att få återfall än de som hade ett svagare uttryck. Vi konkluderar att p53 uttryck, analyserat med immunhistokemi som är en vanlig och tillgänglig metod i kliniken, har potential som en prognosmarkör hos barn med leukemi och att detta borde studeras ytterligare i en större studie med fler patienter.

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8 REFERENCES

1. Kampen, K. R. The discovery and early understanding of leukemia. *Leuk. Res.* **36,** 6–13 (2012).

2. Story, T. H. E., Chronic, O. F. & Leukaemia, M. Historical Review. *Br. J. Haematol.* 2–11 (2000).

3. Pui, C. H. & Evans, W. E. A 50-year journey to cure childhood acute lymphoblastic leukemia. *Semin. Hematol.* **50,** 185–196 (2013).

4. Henig, I. & Zuckerman, T. Hematopoietic Stem Cell Transplantation—50 Years of Evolution and Future Perspectives. *Rambam Maimonides Med. J.* **5,** e0028 (2014).

5. Hunger, S. P. *et al.* Improved Survival for Children and Adolescents With Acute Lymphoblastic Leukemia Between 1990 and 2005 : A Report From the Children ' s Oncology Group. *J. Clin. Oncol.* **30,** 1663–1669 (2012).

6. Pui, C.-H. *et al.* Childhood Acute Lymphoblastic Leukemia: Progress Through Collaboration. *J. Clin. Oncol.* **33,** (2015).

7. Bonaventure, A. *et al.* Worldwide comparison of survival from childhood leukaemia for 1995–2009, by subtype, age, and sex (CONCORD-2): a population-based study of individual data for 89 828 children from 198 registries in 53 countries. *Lancet Haematol.* **4,** e202–e217 (2017).

8. Kogner, P. & Heyman, M. Childhood Cancer Incidence and Survival in Sweden 1984-2010. *Swedish Child. Cancer Regist. Rep.* (2013).

9. Inaba, H., Greaves, M. & Mullighan, C. G. Acute lymphoblastic leukaemia. *Lancet* **381,** 1943–1955 (2013).

10. Winter, S. S. *et al.* Improved Survival for Children and Young Adults With T-Lineage Acute Lymphoblastic Leukemia: Results From the Children's Oncology Group AALL0434 Methotrexate Randomization. *J. Clin. Oncol.* JCO.2018.77.725 (2018). doi:10.1200/JCO.2018.77.7250

11. Chen, B. *et al.* Identification of fusion genes and characterization of transcriptome features in T-cell acute lymphoblastic leukemia. *PNAS* **115,** 373–378 (2018).

12. de Rooij, J., Zwaan, C. & van den Heuvel-Eibrink, M. Pediatric AML: From Biology to Clinical Management. *J. Clin. Med.* **4,** 127–149 (2015).

13. Niemeyer, C. M. & Kratz, C. P. Paediatric myelodysplastic syndromes and juvenile myelomonocytic leukaemia: Molecular classification and treatment options. *Br. J. Haematol.* **140,** 610–624 (2008).

14. Lipka, D. B. RAS-pathway mutation patterns define epigenetic subclasses in juvenile myelomonocytic leukemia. *Nat. Commun.* (2017). doi:10.1038/s41467-017-02177-w

15. Madhusoodhan, P. P., Carroll, W. L. & Bhatla, T. Progress and Prospects in Pediatric Leukemia. *Curr. Probl. Pediatr. Adolesc. Health Care* **46,** 229–241 (2016).

16. Wayne, A. S., Baird, K. & Egeler, R. M. Hematopoietic Stem Cell Transplantation for Leukemia. *Pediatr Clin North Am* **57,** 1–25 (2011).

17. Bhojwani, D., Yang, J. J. & Pui, C.-H. Biology of Childhood Acute Lymphoblastic Leukemia. *Pediatr Clin North Am* **62,** 47–60 (2015).

18. Locatelli, F. *et al.* Outcome of children with high-risk acute myeloid leukemia given autologous or allogeneic hematopoietic cell transplantation in the aieop AML-2002/01 study. *Bone Marrow Transplant.* **50,** 181–188 (2015).

19. Wareham, N. E. *et al.* Outcome of poor response paediatric AML using early SCT. *Eur. J. Haematol.* **90,** 187–194 (2012).

20. Pehlivan, K. C., Duncan, B. B., Lee, D. W. & Lee, D. W. CAR-T Cell Therapy for Acute Lymphoblastic Leukemia : Transforming the Treatment of Relapsed and Refractory Disease. *Curr. Hematol. Malig. Rep.* (2018).

21. Hasle, H. A critical review of which children with acute myeloid leukaemia need stem cell procedures. *Br. J. Haematol.* **166,** 23–33 (2014).

22. Kaspers, G. J. L. & Creutzig, U. Pediatric acute myeloid leukemia: international progress and future directions. *Leuk. Off. J. Leuk. Soc. Am. Leuk. Res. Fund, U.K* **19,** 2025–2029 (2005).

23. Bhojwani, D. & Pui, C.-H. Relapsed childhood acute lymphoblastic leukaemia. *Lancet Oncol.* **14,** e205–e217 (2013).

24. Abrahamsson, J. *et al.* Response-guided induction therapy in pediatric acute myeloid leukemia with excellent remission rate. *J. Clin. Oncol.* **29,** 310–315 (2011).

25. Oskarsson, T. *et al.* Relapsed childhood acute lymphoblastic leukemia in the Nordic countries : prognostic factors , treatment and outcome. *Haematologica* **101,** 68–76 (2016).

26. Pulsipher, M. a., Bader, P., Klingebiel, T. & Cooper, L. J. N. Allogeneic Transplantation for Pediatric Acute Lymphoblastic Leukemia: The Emerging Role of Peritransplantation Minimal Residual Disease/Chimerism Monitoring and Novel Chemotherapeutic, Molecular, and Immune Approaches Aimed at Preventing Relapse. *Biol. Blood Marrow Transplant.* **15,** 62–71 (2009).

27. Horan, J. T. *et al.* Impact of disease risk on efficacy of matched related bone marrow transplantation for pediatric acute myeloid leukemia: The Children's Oncology Group. *J. Clin. Oncol.* **26,** 5797–5801 (2008).

28. Ishaqi, M. K., Afzal, S., Dupuis, A., Doyle, J. & Gassas, A. Outcome of allogeneic hematopoietic stem cell transplantation for children with acute myelogenous leukemia in second complete remission: Single center experience. *Pediatr. Transplant.* **13,** 999–1003 (2009).

29. Lovisa, F. *et al.* Pre- and post-transplant minimal residual disease predicts relapse occurrence in children with acute lymphoblastic leukaemia. *Br. J. Haematol.* **180,** 680–693 (2018).

30. Yanir, A. D. *et al.* Current Allogeneic Hematopoietic Stem Cell Transplantation for Pediatric Acute Lymphocytic Leukemia: Success, Failure and Future Perspectives—A Single-Center Experience, 2008 to 2016. *Biol. Blood Marrow Transplant.* 1–8 (2018). doi:10.1016/j.bbmt.2018.03.001

31. Rujkijyanont, P. *et al.* Risk-adapted donor lymphocyte infusion based on chimerism

and donor source in pediatric leukemia. *Blood Cancer J.* **3,** e137 (2013).

32. Bader, P., Willasch, a & Klingebiel, T. Monitoring of post-transplant remission of childhood malignancies: is there a standard? *Bone Marrow Transplant.* **42 Suppl 2,** S31–S34 (2008).

33. Bader, P. *et al.* Mixed hematopoietic chimerism after allogeneic bone marrow transplantation: the impact of quantitative PCR analysis for prediction of relapse and graft rejection in children. *Bone Marrow Transplant.* **19,** 697–702 (1997).

34. Fossat, C. *et al.* Methodological aspects of minimal residual disease assessment by flow cytometry in acute lymphoblastic leukemia: A french multicenter study. *Cytom. Part B Clin. Cytom.* **88,** 21–29 (2015).

35. Buccisano, F. *et al.* Prognostic and therapeutic implications of minimal residual disease detection in acute myeloid leukemia. *Blood* **119,** 332–341 (2012).

36. Selim, A. G. & Moore, A. S. Molecular Minimal Residual Disease Monitoring in Acute Myeloid Leukemia: Challenges and Future Directions. *J. Mol. Diagnostics* **20,** 389–397 (2018).

37. Pui, C.-H., Robison Leslie L & Look, A. T. Acute Lymphoblastic Leukaemia. *Lancet* **371,** 1030–43 (2008).

38. Eden, T. Aetiology of childhood leukaemia. *Cancer Treat. Rev.* **36,** 286–297 (2010).

39. Petridou, E. T. *et al.* Advanced parental age as risk factor for childhood acute lymphoblastic leukemia: results from studies of the Childhood Leukemia International Consortium. *Eur. J. Epidemiol.* (2018). doi:10.1007/s10654-018-0402-z

40. Somanna, N. K. *et al.* Parental age and childhood cancer risk: A Danish population-based registry study. *Cancer Epidemiol.* **231,** 1130–1141 (2017).

41. Greaves, M. Infection, immune responses and the aetiology of childhood leukaemia. *Nat. Rev. Cancer* **6,** 193–203 (2006).

42. Hwee, J. *et al.* A systematic review and meta-analysis of the association between childhood infections and the risk of childhood acute lymphoblastic leukaemia. *Br. J. Cancer* **118,** 127–137 (2018).

43. Moorman, A. V. *et al.* Prognostic effect of chromosomal abnormalities in childhood B-cell precursor acute lymphoblastic leukaemia: Results from the UK Medical Research Council ALL97/99 randomised trial. *Lancet Oncol.* **11,** 429–438 (2010).

44. Moorman, A. V *et al.* A novel integrated cytogenetic and genomic classification refines risk stratification in pediatric acute lymphoblastic leukemia. *Blood* **124,** 1434–1445 (2018).

45. Toft, N. *et al.* Results of NOPHO ALL2008 treatment for patients aged 1-45 years with acute lymphoblastic leukemia. *Leukemia* **32,** 606–615 (2018).

46. Singh, M. *et al.* High frequency of intermediate and poor risk copy number abnormalities in pediatric cohort of B-ALL correlate with high MRD post induction. *Leuk. Res.* **66,** 79–84 (2018).

47. Stanulla, M. *et al.* IKZF1 plus Defines a New Minimal Residual Disease – Dependent Very-Poor Prognostic Pro fi le in Pediatric B-Cell Precursor Acute Lymphoblastic

Leukemia. *J. Clin. Oncol.* **36,** (2018).

48. Vrooman, L. M. *et al.* Refining risk classification in childhood B acute lymphoblastic leukemia: results of DFCI ALL Consortium Protocol 05-001. *Blood Adv.* **2,** 1449–1458 (2018).

49. Churchman, M. L. *et al.* Germline Genetic IKZF1 Variation and Predisposition to Childhood Acute Lymphoblastic Leukemia. *Cancer Cell* **33,** 937–948.e8 (2018).

50. Mullighan, C. G. Molecular genetics of B-precursor acute lymphoblastic leukemia. *J. Clin. Invest.* **122,** 3407–3415 (2012).

51. Kathiravan, M. *et al.* Deletion of *CDKN2A/B* is associated with inferior relapse free survival in pediatric B cell acute lymphoblastic leukemia. *Leuk. Lymphoma* **0,** 1–9 (2018).

52. Stengel, A., Schnittger, S., Weissmann, S., Kuznia, S. & Kern, W. TP53 mutations occur in 15 . 7 % of ALL and are associated with MYC -rearrangement , low hypodiploidy and a poor prognosis. *Lymphoid Neoplasia* **124,** 251–259 (2014).

53. Holmfeldt, L. *et al.* The genomic landscape of hypodiploid acute lymphoblastic leukemia. *Nat. Genet.* **45,** 242–52 (2013).

54. Forero-Castro, M. *et al.* Mutations in TP53 and JAK2 are independent prognostic biomarkers in B-cell precursor acute lymphoblastic leukaemia. *Br. J. Cancer* **117,** 256–265 (2017).

55. Cavé, H. & Suciu, S. Clinical significance of HOX11L2 expression linked to t (5; 14)(q35; q32), of HOX11 expression, and of SIL-TAL fusion in childhood T-cell malignancies: results of EORTC studies 58881 and 58951. *Blood* **103,** 442–450 (2004).

56. Olshanskaya, Y. *et al.* Clinical significance of cytogenetic changes in childhood T-cell acute lymphoblastic leukemia: results of the multicenter group Moscow–Berlin (MB). *Leuk. Lymphoma* **0,** 1–7 (2018).

57. Breit, T. M. *et al.* Site-specific deletions involving the tal-1 and sil genes are restricted to cells of the T cell receptor alpha/beta lineage: T cell receptor delta gene deletion mechanism affects multiple genes. *J. Exp. Med.* **177,** 965–77 (1993).

58. Carlotti, E. *et al.* Molecular characterization of a new recombination of the SIL / TAL-1 locus in a child with T-cell acute lymphoblastic leukaemia. *Br. J. Haematol.* **118,** 1011–1018 (2002).

59. Ballerini, P. *et al.* Impact of genotype on survival of children with T-cell acute lymphoblastic leukemia treated according to the French protocol FRALLE-93: The effect of TLX3/HOX11L2 gene expression on outcome. *Haematologica* **93,** 1658–1665 (2008).

60. Mansur, M. B. *et al.* SIL-TAL1 fusion gene negative impact in T-cell acute lymphoblastic leukemia outcome. *Leuk. Lymphoma* **50,** 1318–25 (2009).

61. Assumpção, J. G. *et al.* Gene rearrangement study for minimal residual disease monitoring in children with acute lymphocytic leukemia. *Rev. Bras. Hematol. Hemoter.* **35,** 337–342 (2013).

62. D'Angio, M. *et al.* Clinical features and outcome of SIL/TAL1-positive T-cell acute lymphoblastic leukemia in children and adolescents: a 10-year experience of the AIEOP group. *Haematologica* 344–347 (2015).

63. van Dongen, J. J. *et al.* Standardized RT-PCR analysis of fusion gene transcripts from chromosome aberrations in acute leukemia for detection of minimal residual disease. Report of the BIOMED-1 Concerted Action: investigation of minimal residual disease in acute leukemia. *Leukemia* **13,** 1901–28 (1999).

64. Furness, C. L. *et al.* The subclonal complexity of STIL-TAL1+ T-cell acute lymphoblastic leukaemia. *Leuk. 2018* 1 (2018). doi:10.1038/s41375-018-0046-8

65. Mansur, M. B. *et al.* Distinctive genotypes in infants with T-cell acute lymphoblastic leukaemia. *Br. J. Haematol.* **171,** 574–584 (2015).

66. Asnafi, V. *et al.* Age-related phenotypic and oncogenic differences in T-cell acute lymphoblastic leukemias may reflect thymic atrophy. *Blood* **104,** 4173–4180 (2004).

67. Paganin, M. *et al.* The presence of mutated and deleted PTEN is associated with an increased risk of relapse in childhood T cell acute lymphoblastic leukaemia treated with AIEOP-BFM ALL protocols. *Br. J. Haematol.* **182,** 705–711 (2018).

68. Carrasco Salas, P. *et al.* The role of CDKN2A/B deletions in pediatric acute lymphoblastic leukemia. *Pediatr. Hematol. Oncol.* **33,** 415–422 (2016).

69. Tao, Y.-F. *et al.* Analyzing the gene expression profile of pediatric acute myeloid leukemia with real-time PCR arrays. *Cancer Cell Int.* **12,** 40 (2012).

70. Pastor, V. *et al.* Mutational landscape in children with myelodysplastic syndromes is distinct from adults: specific somatic drivers and novel germline variants. *Leukemia* **31,** 759–762 (2017).

71. Schwartz, J. R. *et al.* The genomic landscape of pediatric myelodysplastic syndromes. *Nat. Commun.* 1–9 (2017). doi:10.1038/s41467-017-01590-5

72. Suttorp, M., Eckardt, L., Tauer, J. T. & Millot, F. Management of chronic myeloid leukemia in childhood. *Curr. Hematol. Malig. Rep.* **7,** 116–124 (2012).

73. Greaves, M. F. & Wiemels, J. Origins of chromosome translocations in childhood leukaemia. *Nat. Rev. Cancer* **3,** 639–649 (2003).

74. Marshall, G. M. *et al.* The prenatal origins of cancer. *Nat. Rev. Cancer* **14,** 277–289 (2014).

75. Tavian, M., Cortés, F., Charbord, P., Labastie, M. C. & Péault, B. *Emergence of the haematopoietic system in the human embryo and foetus. Haematologica* **84 Suppl E,** (1999).

76. Tavian, M. & Peault, B. Embryonic development of the human hematopoietic system. *Int. J. Dev. Biol.* **49,** 243–250 (2005).

77. Holt, P. G. & Jones, C. A. The development of the immune system during pregnancy and early life. *Allergy* **55,** 688–697 (2000).

78. Gale, K. B. *et al.* Backtracking leukemia to birth: identification of clonotypic gene fusion sequences in neonatal blood spots. *Proc. Natl. Acad. Sci. U. S. A.* **94,** 13950–13954 (1997).

79. Maia, A. T. *et al.* Molecular tracking of leukemogenesis in a triplet pregnancy. *Blood* **98,** 478–482 (2001).

80. McHale, C. M. *et al.* Prenatal origin of TEL-AML1-positive acute lymphoblastic leukemia in children born in California. *Genes Chromosom. Cancer* **37,** 36–43 (2003).

81. Ma, Y. *et al.* Developmental timing of mutations revealed by whole-genome sequencing of twins with acute lymphoblastic leukemia. *Proc. Natl. Acad. Sci. U. S. A.* **110,** 7429–33 (2013).

82. Cazzaniga, G. *et al.* Developmental origins and impact of BCR-ABL1 fusion and IKZF1 deletions in monozygotic twins with Ph + acute lymphoblastic leukemia. *Lymphoid Neoplasia* **118,** 5559–5564 (2014).

83. Ford, a M. *et al.* Monoclonal origin of concordant T-cell malignancy in identical twins. *Blood* **89,** 281–5 (1997).

84. Fasching, K. *et al.* Presence of clone-specific antigen receptor gene rearrangements at birth indicates an in utero origin of diverse types of early childhood acute lymphoblastic leukemia. *Blood* **95,** 2722–2724 (2000).

85. Eden, O. B. *et al.* Prenatal origin of acute lymphoblastic leukaemia in children. *Lancet* **354,** 1499–1503 (1999).

86. Hjalgrim, L. L. *et al.* Presence of clone-specific markers at birth in children with acute lymphoblastic leukaemia. *Br. J. Cancer* **87,** 994–999 (2002).

87. Lukes, J. *et al.* Two novel fusion genes, *AIF1L-ETV6* and *ABL1-AIF1L* , result together with *ETV6-ABL1* from a single chromosomal rearrangement in acute lymphoblastic leukemia with prenatal origin. *Genes, Chromosom. Cancer* 1–7 (2018). doi:10.1002/gcc.6

88. Ford, a M. *et al.* Fetal origins of the TEL-AML1 fusion gene in identical twins with leukemia. *Proc. Natl. Acad. Sci. U. S. A.* **95,** 4584–4588 (1998).

89. Morak, M. *et al.* Clone-specific secondary aberrations are not detected in neonatal blood spots of children with ETV6-RUNX1-positive leukemia. *Haematologica* **98,** e108–e110 (2013).

90. Mori, H. *et al.* Chromosome translocations and covert leukemic clones are generated during normal fetal development. *Proc. Natl. Acad. Sci. U. S. A.* **99,** 8242–8247 (2002).

91. Zuna, J. *et al.* ETV6/RUNX1 (TEL/AML1) is a frequent prenatal first hit in childhood leukemia. *Blood* **117,** 368–369 (2011).

92. Lausten-Thomsen, U. *et al.* Prevalence of t(12;21)[ETV6-RUNX1]-positive cells in healthy neonates. *Blood* **117,** 186–189 (2011).

93. Barbany, G. *et al.* The ETV6/RUNX1 fusion transcript is not detected in RNA isolated from neonatal dried blood spots from children later diagnosed with the corresponding leukemia. *Leuk. Lymphoma* **54,** 2742–2744 (2013).

94. Schäfer, D. *et al.* 5 % of healthy newborns have an ETV6-RUNX1 fusion as revealed by DNA-based GIPFEL screening. *Blood* (2018). doi:10.1182/blood-2017-09-808402

95. Renate Panzer-Grümayer, E. *et al.* Nondisjunction of chromosomes leading to

hyperdiploid childhood B-cell precursor acute lymphoblastic leukemia is an early event during leukemogenesis. *Blood* **100,** 347–349 (2002).

96. Bateman, C. M. *et al.* Evolutionary trajectories of hyperdiploid ALL in monozygotic twins. *Leukemia* **29,** 1–8 (2014).

97. Maia, a T. *et al.* Prenatal origin of hyperdiploid acute lymphoblastic leukemia in identical twins. *Leukemia* **17,** 2202–2206 (2003).

98. Wiseman, D. H. Donor Cell Leukemia : A Review. *Biol. Blood Marrow Transplant.* **17,** 771–789 (2011).

99. Škorvaga, M. *et al.* Backtracked analysis of preleukemic fusion genes and DNA repair foci in umbilical cord blood of children with acute leukemia. *Oncotarget* **9,** 19233–19244 (2018).

100. Wiley, Ó. J. *et al.* Donor cell-derived acute myeloid leukemia after second allogenic cord blood transplantation in a patient with Fanconi anemia. *Pediatr. Transplant.* **16,** 241–245 (2012).

101. Andrew C. Dietz, Todd E. DeFor, Claudio G. Brunstein, and J. E. W. J. Donor-derived myelodysplastic syndrome and acute leukaemia after allogeneic haematopoietic stem cell transplantation: incidence, natural history and treatment response. *Br. J. Cancer* **166,** 209–212 (2015).

102. Pant, V., Quintás-Cardama, A. & Lozano, G. The p53 pathway in hematopoiesis: Lessons from mouse models, implications for humans. *Blood* **120,** 5118–5127 (2012).

103. Stengel, A. *et al.* The impact of TP53 mutations and TP53 deletions on survival varies between AML, ALL, MDS and CLL—an analysis of 3307 cases. *Leukemia* **31,** 705–711 (2016).

104. Middeke, J. M. *et al.* TP53 mutation in patients with high-risk acute myeloid leukaemia treated with allogeneic haematopoietic stem cell transplantation. *Br. J. Haematol.* **172,** 914–922 (2016).

105. Hof, J. *et al.* Mutations and deletions of the TP53 gene predict nonresponse to treatment and poor outcome in first relapse of childhood acute lymphoblastic leukemia. *J. Clin. Oncol.* **29,** 3185–3193 (2011).

106. Wada, M. *et al.* Analysis of p53 mutations in a large series of lymphoid hematologic malignancies of childhood. *Blood* **82,** 3163–3169 (1993).

107. Krentz, S. *et al.* Prognostic value of genetic alterations in children with first bone marrow relapse of childhood B-cell precursor acute lymphoblastic leukemia. *Leukemia* 295–304 (2012). doi:10.1038/leu.2012.155

108. Qian, M. *et al.* TP53 germline variations influence the predisposition and prognosis of b-cell acute lymphoblastic leukemia in children. *J. Clin. Oncol.* **36,** 591–599 (2018).

109. Irving, J. A. E. *et al.* Integration of genetic and clinical risk factors improves prognostication in relapsed childhood B-cell precursor acute lymphoblastic leukaemia. *Blood* **128,** 911–923 (2016).

110. Addeo, R. *et al.* Prognostic Role of bcl-x and p53 in Childhood Acute Lymphoblastic Leukemia (ALL). *Cancer Biol. Ther.* **4047,** (2005).

111. Gustafsson, B., Christenson, B., Hjalmar, V. & Winiarski, J. Cellular expression of MDM2 and p53 in childhood leukemias with poor prognosis. *Med. Pediatr. Oncol.* **34,** 117–24 (2000).

112. Liang, D. *et al.* Cooperating gene mutations in childhood acute myeloid leukemia with and DNMT3A. *Blood* **121,** 2988–2995 (2013).

113. Kattamis, A. C., Tsangaris, G. T., Vamvoukakis, J., Moschovi, M. & Grafakos, S. Detection of Minimal Residual Disease by Mutant p53 Immunocytochemistry in Acute Myelogenous Leukemia. *Med. Pediatr. Oncol.* **156,** 153–156 (2000).

114. Miyauchi, J. *et al.* Abnormalities of the p53 gene in juvenile myelomonocytic leukaemia. *Br. J. Haematol.* **106,** 980–6 (1999).

115. Saito, S. *et al.* Genetic analysis of TP53 in childhood myelodysplastic syndrome and juvenile myelomonocytic leukemia. *Leuk. Res.* **35,** 1578–1584 (2011).

116. Silveira, C. G. T. *et al.* New recurrent deletions in the PPARγ and TP53 genes are associated with childhood myelodysplastic syndrome. *Leuk. Res.* **33,** 19–27 (2009).

117. Jekic, B. *et al.* Lack of TP53 and FMS gene mutations in children with myelodysplastic syndrome. *Cancer Genet. Cytogenet.* **166,** 163–165 (2006).

118. Diaz-Moralli, S., Tarrado-Castellarnau, M., Miranda, A. & Cascante, M. Targeting cell cycle regulation in cancer therapy. *Pharmacol. Ther.* **138,** 255–271 (2013).

119. Peterson, L. F., Yan, M. & Zhang, D. The p21 Waf1 pathway is involved in blocking leukemogenesis by the t (8 ; 21) fusion protein AML1-ETO. *Blood* **109,** 4392–4399 (2007).

120. Zhao, Y. *et al.* Downregulation of p21 in Myelodysplastic Syndrome Is Associated With p73 Promoter Hypermethylation and Indicates Poor Prognosis. *Am. J. Clin. Pathol.* **140,** 819–827 (2013).

121. Salmena, L., Carracedo, A. & Pandolfi, P. P. Tenets of PTEN Tumor Suppression. *Cell* **133,** 403–414 (2008).

122. Gutierrez, A. *et al.* High frequency of PTEN, PI3K, and AKT abnormalities in T-cell acute lymphoblastic leukemia. *Blood* **114,** 647–650 (2009).

123. Barbi, M. *et al.* Diagnosis of congenital cytomegalovirus infection by detection of viral DNA in dried blood spots. *J. Clin. Virol.* **35,** 206–9 (1996).

124. Priftakis, P. *et al.* Human polyomavirus DNA is not detected in Guthrie cards (dried blood spots) from children who developed acute lymphoblastic leukemia. *Med. Pediatr. Oncol.* **40,** 219–223 (2003).

125. Gustafsson, B. *et al.* Adenovirus DNA is detected at increased frequency in Guthrie cards from children who develop acute lymphoblastic leukaemia. *Br. J. Cancer* **97,** 992–994 (2007).

126. Honkaniemi, E. *et al.* Adenovirus DNA in Guthrie cards from children who develop acute lymphoblastic leukaemia (ALL). *Br. J. Cancer* **102,** 796–798 (2010).

127. Gustafsson, B. *et al.* KI, WU, and Merkel cell polyomavirus DNA was not detected in guthrie cards of children who later developed acute lymphoblastic leukemia. *J. Pediatr. Hematol. Oncol.* **34,** 364–367 (2012).

128. Saft, L. *et al.* P53 Protein Expression Independently Predicts Outcome in Patients With Lower-Risk Myelodysplastic Syndromes With Del(5Q). *Haematologica* **99,** 1041–1049 (2014).

129. Jädersten, M. *et al.* TP53 mutations in low-risk myelodysplastic syndromes with del(5q) predict disease progression. *J. Clin. Oncol.* **29,** 1971–1979 (2011).

130. Zuna, J. *et al.* Implications of delayed bone marrow aspirations at the end of treatment induction for risk stratification and outcome in children with acute lymphoblastic leukaemia. *Br. J. Haematol.* **173,** 742–748 (2016).

131. Vogelstein, B., Lane, D. & Levine, a J. Surfing the p53 network. *Nature* **408,** 307–310 (2000).